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## Selection System

The present invention relates to a screening system useful for screening repertoires of DNA binding domains. In particular the invention relates to a screening system based on transcriptional activators of bacterial σ54-dependent promotors.

The majority of proteins involved in cellular functions do so by interacting with other proteins or nucleic acid sequences within the cell. Several approaches have been described that allow an in vivo selection of relevant clones expressing a particular protein or DNA-binding activity. Arguably the most powerful approach are the yeast one- and two hybrid system (Fields & Song 89) for the screening of protein-DNA, respectively protein-protein interactions. However, the two-hybrid system requires an eucaryotic host and consequently the diversity that can be screened is limited. Furthermore the system notoriously suffers from an abundance of false positives.

Larger molecular repertoires can be prepared in bacterial hosts and a number of bacterial systems for the screening of protein-protein and protein-DNA interactions have also been reported. Two systems have been put forward in which the polypeptide chain of an enzyme is expressed in two parts fused to two proteins X and Y and in which cognate X-Y interaction reconstitutes function of the enzyme (Karimova 98, Pelletier 98).

Several in vivo screens for DNA-binding proteins have also been reported (reviewed in Mossing 91, Elledge 89). Each of these methods involves the blockage of a hybrid σ70 promotor by the DNA binding protein. Repression of the promotor either prevents the production of conditionally toxic gene or alleviates repression of an antibiotic gene by transcriptional interference. The transcriptional interference assay (Elledge 89) has been used successfully in one case to select DNA binding proteins with altered specificity (Sera & Schultz 96).

Transcriptional activators of σ54 dependent promotors have been called bacterial enhancers because their mechanism of activation is superficially similar to the activation of transcription by enhancer proteins in eucaryotes (Kustu 91). Unlike σ70 promotors at which the RNA polymerase is bound in an active form and is largely controlled by repression, the σ54 RNA polymerase holoenzyme is transcriptionally incompetent. Its conversion into an active form is catalysed by the enhancer protein coupled to hydrolysis of ATP. This unusual mechanism accounts for the low level of background transcription and the normous difference ($10^4$-$10^5$) between on and off states in the strongest σ54

promotors effected by a single factor. By comparison activators of σ70 promotors such as CAP or λcI increase transcription levels usually by less than 10-fold.

We provide herein a novel screening system which is based on transcriptional activators of σ54-based promotors.

Preferred embodiments of the invention are described in the appended claims.

The Nif family of bacterial enhancers regulate expression of nitrogenase components from σ54 promotors in nitrogen-fixing bacteria, and are inhibited by NifL (Austin 94). In bacteria lacking NifL, NifA is constitutionally active. NifA is modular in architecture and we found that this allows for the swapping of the natural DNA-binding domain (DBD) for extraneous DBDs. Such NifA-DBD chimaeras are inactive on the wild type promotor, but activate transcription from hybrid-promotors bearing their cognate target sequences. We propose to prepare and select libraries of NifA-hybrids together with libraries of hybrid promotors. "Crossing" of hybrid libraries will use combinatorial infection, which has been employed successfully to generate very large antibody libraries (Griffith et al 94).

"Generic" libraries are preferably based on a known DBD architectures (e.g. basic leucine zipper, bZIP) and may be derived using PCR amplification with "family-specific" primers. Such libraries may be crossed with hybrid-promotors bearing defined target sequences or libraries of target sequences. In addition to providing information on the distribution of members of the family in a given genome, such libraries may be used to identify and study proteins or molecular compounds that modify DNA interaction within a family of DBDs, for example Tax (from HTLV-1) in the case of bZIP proteins.

In an alternative embodiment, they may also be used to select DBDs which conditionally bind their target sequence only in the presence of other factors such as protein cofactors or small molecular compounds, for example drugs that intercalate into DNA or alter the degree of supercoiling. The system can also be used "in reverse" i.e. to select proteins or molecular compounds that disrupt a particular DNA-protein interaction or to select DBDs that do not bind a particular target sequence or library thereof.

More advanced libraries are preferably derived directly from genomic DNA or cDNA libraries and selected on hybrid promotors bearing a repertoire of target sequences, comprising either a stretch of randomized sequence or a library of inserts derived from fragmented genomic DNA. Data obtained in this way allows the compilation of a genomic directory of DBDs and the building of a promotor-DBD-interaction map.

The NifA chimera also offer the opportunity to better understand aspects of the process of transcriptional activation at σ54 promotors. It has been known for some time that binding of the target sequence together with ATP binding promotes oligomerization of NifA. It is believed that it is the oligomer which contacts the polymerase and catalyses the ATP-driven isomerization of the polymerase holoenzyme. Taking advantage of the superactivation effect described above it may be possible to address questions such as which components of the oligomer (e.g. the DNA-bound NifA vs. NifAΔC), which are contacting the polymerase and/or coupling ATP hydrolysis to transcriptional activation etc. Furthermore, usage of NifAΔC cofactors from different species (possibly together with their diversification by PCR shuffling) may allow identification of the sequence regions critical for transcriptional activation and may allow a "maturation" of the NifAΔC coactivator. Indeed, we found the NifA from *K. pneumoniae* to be a superior cofactor to *A. vinelandii* NifA. Finally, it may be possible to use chimera of a known DBD (e.g. GCN4) and a cDNA library as a procaryotic "enhancer" trap, to isolate σ54 activators on a genome-wide scale.

Protein-protein interactions may also be selected using the NifA system. Two hybrid proteins are generated: a bait-hybrid comprising protein X fused to a DNA-binding domain and a prey-hybrid comprising protein Y fused to the NifA activation and sensor domains with cognate interaction of X and Y reconstituting the transcriptional activator. The NifA bacterial two-hybrid system could be used for the generation of interaction matrices between cDNA libraries. Ultimately such interaction matrices may yield an interaction map of the proteins of an organism, and may provide an alternative to the yeast two hybrid system.

Systems based on σ54 have a number of advantages over the other systems that are available, e.g. the conceptually similar yeast one-hybrid system. A bacterial host allows substantially larger repertoires to be obtained and thus a much larger molecular diversity to be screened. In particular, using combinatorial infection, our system should allow the "crossing" of both NifA-chimera repertoires with libraries of hybrid reporter constructs, thus permitting coevolution of DBD and recognition sites , or coselection of DBD and target sites from genomic libraries.

Because selection in the σ54-based system is based on a positive readout, i.e. activation of transcription it should be less prone to false positives than other approaches relying on the inhibitory effect of the expressed DBD like the transcription interference assay (Elledge 89). In vivo selection in general may result in the selection of novel DBD that are more

attuned to working under realistic conditions, including supercoiling of the recognition site, presence of a large excess of chromosomal DNA and high protein concentration. Another advantage of our system may the act that extremely low level of expression appears to be sufficient to affect maximum activation of transcription. This may be particularly helpful in the case of DBDs that are prone to aggregation.

The σ54-based systems of the present invention may be further adapted to take into account potential disadvantages of bacterial expression. For instance, E. coli expression may be suboptimal for large eucaryotic transcription factors. However, large eucaryotic proteins can usually be split into smaller domains which retain function and are usually readily expressed in E. coli.

## Examples

NifA from *A. vinelandii* is a well-studied member of the family of bacterial enhancers and it is a positive regulator of the expression of nitrogenase components in diazotrophs. It is inhibited by NifL in response to the presence of oxygen or ammonia. When expressed in E. coli, which lacks endogenous NifL or an equivalent, NifA is constitutionally active. Because of the highly conserved nature of the activation mechanism of σ54 RNA polymerase, NifA is a very strong activator of transcription in *E. coli.*

Like other members of the family of bacterial enhancer proteins, NifA is modular in architecture, both structurally and functionally, comprising 3 domains, a N-terminal sensor domain , a central activation domain (AD), and a C-terminal DNA binding domain (DBD). The central activation domain (AD) can activate transcription independent of DNA binding if overexpressed. Thus the DBD's function appears to be primarily to increase the Activator domain's concentration in the promotor proximity.

We have exploited the modularity of the enhancer structure and swapped the natural NifA DNA binding domain (DBD) for extraneous DBDs and libraries thereof. Here we describe the activity of these NifA-chimeras in the activation of transcription from the s54 dependent promotor nifH and hybrids thereof.

## Materials & Methods

### Media & Reagents

2xTY, MacConkey agar are described elsewhere (Miller 72 ). Antibiotics were used at the following concentrations: Ampicilline 0.1 mg/ml, Chloramphenicol 10μg/ml,

Streptomycin 25μg/ml. Min-lac medium was essentially M9 medium (Sambrook) supplemented with 1mM $MgSO_4$, 20μM $CaCl_2$, 2% (w/v) lactose, 2mg/ml casamino acids, 40μg/ml L-tryptophan, 5μg/ml thiamine and appropriate antibiotics. Min-lacX plates where essentially M9 plates supplemented 2% lactose, appropriate antibiotics and 40μg/ml X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside).

**Strains**

TG1ΔK was derived from TG1 (Gibson 94) using the genome integration strategy of (Haldimann 96). Briefly, NifA(*K. pneumoniae*) residues 1-462 was amplified using Pfu polymerase (Stratagene) and primers 1, 2, cut with NdeI and BamHI and cloned into the genome targeting suicide vector pSK50D-uidA2 (Haldimann 96) and transformed into the $Pir^+$ host strain BW23473 (Metcalf 96). Vectors were isolated and transformed into the $Pir^-$ strain TG1 harbouring the plasmid pINT-ts (Hasan 94). Chromosomal integration was induced by a temperature shift to 42°C, which leads to expression of λ integrase from pINT-ts and simultaneously stops its replication. Integrants where identified by Kanamycin resistance and screened for Nif coactivation. Once obtained TG1ΔK was grown routinely without antibiotic selection.

**Constructs**

Chimera constructs were based on pDB737 (Austin 95) encoding NifA (*A. vinelandii*) under the control of the T7 promotor in the plasmid pT7-7 (Tabor & Richardson 85). Expression was by leakiness of the T7 promotor. Chimeras were constructed taking advantage of an unique BanII cutting site, in the linker region between the central domain of NifA and the DBD. GCN4 was amplified using Pfu polymerase (Stratagene) and primers 3, 4, 5 and 6, cut with BanII and Hind3 and ligated into pDB737 cut with BanII and Hind3. ERDBD was amplified using primers 7, 8 and 9, cut with BanII and BamHI and ligated into pDB737 cut with BanII and BamHI. Myn was amplified using primers 10 and 11, cut with SalI and Hind3 and ligated into pDB737 cut with SalI and Hind3. The vector p737S1 is derived from pDB737 by replacing the bla gene with aadA conferring streptomycin resistance as the insertion of a fl phage origin for packaging of the vector into filamentous phage particles. Briefly, aadA, was amplified using primers 12, 13 and cut with AatII and ScaI and ligated with pDB737 cut with AatII ad DraIII. The resulting vector p737S was cut with AatII, ClaI. flori was amplified using primers 14, 15 and cloned into the cut p737S to give p737S1. NifA-X chimera were transferred from pDB737 to p737S1 by digestion with NdeI , Hind3 (BamHI for NifA-ERDBD).

Reporter constructs were derived from pACYC184 and the vector pMB1 (Buck 86). Briefly the lac-operon (lacZYA) was amplified with primers 16, 17 and cut with BamHI, MscI. the nifH promotor segment from pMB1 was amplified with primers 18, 19 and cut with Hind3, BamHI and the 2 fragments simultaneously ligated with pACYC184 cut with Hind3 and BsaAI to give pMB3. flori was amplified with primers 20, 21, and cut with NheI and XmnI and ligated into pMB3 cut both NheI, XmnI to give pMB31.

**Selection and screening**

Cells were cotransformed either by simultaneous or sequential electroporation with an expressor construct and a reporter construct and grown ON with appropriate antibiotic selection at 34°C in M9-lac and plated out. β-gal expression was scored either on MacConkey or Minlac-X-gal indicator plates or by ONPG enzyme assay of selelcted colonies (see below).

**Enzyme assay**

ONPG assays were used to measure β-gal activity were essentially as described by (Kolmar 96). Briefly, 20μl of an overnight culture is tranferred to a micrototer well and 100μl of chloroform saturated Z-buffer (100mM $NaHPO_4$, 1mM KCL, 1mM $MgSO_4$, 50mM β-mercaptoethanol, pH 7.0 (Miller 72) was added and the optical desnity at 600nm determined using an ELISA reader. Cells were lyzed by addition of 50μl Z-buffer with 0.4% (w/v) SDS and incubated at 30°C for 10 min. 50μl of Z-buffer with 4mg/ml O-nitrophenyl-β-D-galactopyranoside were added and the optical density at 420nM was recorded automatically every 15s over a period of 60min. Specific β-gal activity was calculated from the $V_{max}$ as in (Miller 72).

**Example 1: NifA chimera with extraneous DNA binding domains activate transcription but only from promotors with a cognate recognition site**

To investigate in what way transcription activation by NifA was dependent on the NifA DNA binding domain (DBD) and on native nif promotor structure, we prepared NifA-chimeras in which NifA DNA binding domain (DBD) had been replaced by extraneous DBDs of diverse structural architecture. Initially we explored DBDs, which like the NifA wt DBD bind to symmetrical DNA recognition sequences such as the basic leucine zipper (bZIP) DBD of the yeast transcription factor GCN4, the Zn-finger domain of the human estrogen receptor DNA binding domain (ERDBD) and the helix-loop-helix (HLH) DBD

from the human transcription factor Max (Myn) and determined their capacity to activate transcription of a lacZ reporter gene in vivo from a hybrid nifH promotor, in which the NifA UAS had been deleted and replaced by recognition sites for the extraneous DBDs.

In order to simplify comparison of transcription activation by NifA chimeras with activation by wt NifA, all reporter constructs had a single DNA recognition site. The wt nifH promotor UAS contains three bona fide NifA recognition sites. Deletion of the two sites more distal to the promotor however did not appear to reduce transcription activation in our reporter under conditions tested.

Transcription activation by NifA-chimeras was specific in that they only activated lacZ expression from hybrid-promotors bearing their cognate recognition sequences but not from control reporter constructs bearing wild type UAS or a non-cognate site. In analogy to wt NifA the presence of two or more recognition sites (in phase, see below) did not increase activation by the Nif-GCN4 chimera.

Activity was also dependent on the phasing of the recognition site with respect to the promotor: when the symmetric ATF/CREB recognition site for GCN4 was offset in increments of 1 bp, optimal activity was observed when the ATF/CREB was centred on the same bp as the symmetric wt UAS. Presumably efficient contact with the RNA Pol holoenzyme requires that the activator be bound on the right face of the DNA.

Transcription activation by NifA-chimeras appears to preserve fine specificity of isolated DBDs. Wild-type GCN4 binds with equal affinity to the symmetric ATF/CREB site as well as to the pseudo-symmetric AP-1 site. Indeed, the NifA-GCN4 chimera showed identical levels of transcription activation in reporter constructs with either of these sites. A NifA-ERDBD chimera showed strong activity on a reporter with its cognate ERE site but no activity above background levels with reporters bearing the similar GRE recognition site for the closely related glucocorticoid receptor DBD.

**Example 2: Coexpression of NifA with NifA-chimeras boosts specific transcription activation by NifA chimeras in a specific and DNA independent manner**

The level of transcription activation by the NifA-GCN4 and NifA-ERDBD chimeras was lower (ca. 10%) than for wt NifA. However, near wt levels of activity (80%) were reached when wt NifA was coexpressed within the same cell as a "coactivator".

The coactivation was independent of DNA binding as a NifA variants in which the DBD had been deleted (NifAΔC) was found to be just as active as wt NifA. On the other coexpression of an isolated NifA central domain (both the DBD as well as the N-terminal sensor domain deleted (NifAΔNC)) failed to coactivate. NifA derived from different species showed greatly variable efficiencies as coactivators. NifA variants from K. pneumonia (NifA Kp, NifAΔC Kp) being almost three times as effective as NifA, while NifA variants from Rhizobium (NifA Rh1, NifA Rh2) were poorly active as coactivators.

The coactivator effect was found to enhance only specific transcription activation but not background levels of transcription from promotors with non-cognate recognition sites. We therefore constructed an E. coli strain, expressing NifAΔC Kp (the *K. pneumoniae* NifA with its DBD deleted) from a weak promotor (phoB) from the chromosome (TG1:ΔK).

The coactivation effect has analogies in eucaryotic transcription , for example the enhancer Sp1, in which isolated Sp1 activation domains can stimulate transcriptional activation by the DNA binding-form of Sp1, a phenomenon termed "superactivation".

**Example 3: Tethering of NifA chimera at the UAS is sufficient for activation, but strong activation requires correct positioning**

We also investigated transcription activation by NifA-chimeras with asymmetrical recognition sites such as the classic Zn-finger Zif268 as well as the DBD from p53. Both NifA-Zif268 and NifA-p53 chimeras activated transcription, but only at low levels (2 - 5-fold above the background). However, when the Zif recognition site was duplicated, to give a symmetric palindromic site transcription activation increased substantially. Non palindromic duplication of the recognition site in tandem did not increase activation.

Thus while simple tethering is sufficient for some activation, only bipartite binding appears to give a strong activation. Presumably, tethering only leads to an approximate positioning of the activation domain with respect to the RNA polymerase holoenzyme, thereby reducing the likelihood of a productive interaction.

**Example 4: Selection of active NifA-chimeras by lac complementation**

Using expression of the lac operon (lacZYA) from our reporter construct as the read-out of transcription activation allows the selection of active NifA-chimera on the basis of metabolic complementation of a Δlac strain, with lactose as the only carbon source. Initially we spiked populations of NifA-ERDBD with NifA-GCN4 at the ratios $1/10^4$, $1/10^6$ in the presence of the GCN4 cognate reporter ATF/CREB-nifH and grew populations overnight in minimal medium supplied with lactose. Pre- and post selection populations were scored by plating on MacConkey-lactose plates as well as by PCR screening. The results are summarised in Table 1. Selection factors of up to 10,000 -fold per round were observed.

In order to determine to what degree the basic region of bZIP proteins is suited as a general framework of DNA recognition we prepared a library of mutant GCN4 bZIP DBDs ($2x10^7$ mutants) in which 5 key residues of DNA interaction are randomized and selected it against the natural ATF/CREB recognition site. After just one round of selection a majority of colonies contained active NifA-GCN4 mutants as judged by lacZ expression.

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**Claims**

1) A method for the screening and selection of protein-DNA interactions and protein-protein interactions comprising a chimeric transcriptional activator of σ54 promotors.

2) A method for the screening and selection of protein-DNA interactions and protein-protein interactions comprising a chimeric constitutionally active transcriptional activator of σ54 promotors.

3) A method for the screening and selection of protein-DNA interactions and protein-protein interactions as in 1) comprising a chimeric transcriptional activator of σ54 promotors in which activation resulting from cognate interactions is enhanced by the coexpression of a full length or variant form of the σ54 transcriptional activator.

4) A method for the screening and selection of protein-DNA interactions in bacteria comprising a chimeric transcriptional activator comprising a transcription activating sequence and

a) a DNA binding sequence
b) a domain with DNA binding activity
c) a library of sequences or domains with DNA binding activity

5) A method for the screening and selection of protein-protein interactions in bacteria comprising a chimeric transcriptional activator comprising a transcription activating sequence and

a) a protein binding sequence
b) a domain with protein binding activity
c) a library of sequences or domains with protein binding activity
d) a library of random peptides

and a chimeric DNA binding protein comprising a DNA binding domain and

a) a protein binding sequence
b) a domain with protein binding activity
c) a library of sequences or domains with protein binding activity
d) a library of random peptides

6) A method for the screening and selection of protein-DNA interactions in bacteria comprising a chimeric transcriptional activator as in 4) and a hybrid σ54 promotor

controlling directly or indirectly the expression of one or more reporter genes conferring a selectable phenotype. The hybrid σ54 promotor comprises a σ54 binding site, and

a) a binding site for integration host factor
b) a binding site for a DNA bending protein
c) an intrinsically bent DNA sequence

as well as

a) a recognition sequence for the enhancer (UAS)
b) a recognition sequence for an extraneous DNA binding protein
c) a library of recognition sites
d) a stretch of random DNA sequence

7) A method for the screening and selection of protein-protein interactions in bacteria comprising a chimeric transcriptional activator as in 5) and a hybrid σ54 promotor controlling directly or indirectly the expression of a reporter gene conferring a selectable phenotype. The hybrid σ54 promotor comprises a σ54 binding site, and

a) a binding site for integration host factor
b) a binding site for a DNA bending protein
c) an intrinsically bent DNA sequence

as well as

a) a recognition sequence for the enhancer (UAS)
b) a recognition sequence for an extraneous DNA binding protein

8) A method for the screening and selection of protein-DNA interactions and protein-protein interactions in bacteria comprising a chimeric NifA.

9) A method for the screening and selection of protein-DNA interactions and protein-protein interactions as in 1) comprising a chimeric NifA in which activation resulting from cognate interactions is enhanced by the coexpression of a full length or variant form of NifA.

10) A method for the screening and selection of protein-DNA interactions and protein-protein interactions as in 1) comprising a chimeric NifA from *Azotobacter vinelandii* in which activation resulting from cognate interactions is enhanced by the coexpression of a full length or variant form of NifA from *Klebsiella pneumoniae.*

11) A DNA sequence; a vector or a linear segment of DNA comprising a hybrid σ54 promotor as in 5) in which the promotor is the nifH promotor from *A. vinelandii.* and the reporter genes are

a) metabolic markers such as the lac operon (lacZ, lacY and lacA)

b) conferring a fluorescent phenotype, such as GFP

c) conferring antibiotic resistance such as Zeo

d) conferring another selectable property

12) A method for the screening and selection of protein-DNA interactions and protein-protein interactions as in 1), 2) and 3) in which selections are carried out in the presence of molecular compounds that modify protein-DNA interactions, for example by altering the structure of the DNA binding protein (e.g. Tax for bZIP proteins ) or the DNA structure (e.g. through intercalation or affecting supercoiling) or modify protein-protein interactions (e.g. allosteric effects, creation of new binding surfaces).

13) A method for the screening of target ligands in which selections are carried out in the presence of target ligands hat modify protein-DNA and protein-protein interactions.

14) A method for the screening and selection of protein-DNA interactions and protein-protein interactions as in 1), 2) and 3) in which selections are carried out in vivo (in bacteria) or in vitro (in vitro transcription and translation ).

15) A method for the screening and selection of protein-DNA interactions and protein-protein interactions as in 1), 2) and 3) in which selections are carried out in E. coli comprising a chimeric NifA as in 10) and a hybrid σ54 promotor as in 11).

16) A kit for research purposes comprising a chimeric transcriptional activator of σ54 promotors and a wild-type or hybrid σ54 promotor or libraries thereof.

17) A method for the screening and selection of activators of σ54 promotors (enhancer trap) comprising hybrid σ54 promotor comprising the target site for a known DBD (e.g. GCN4) as well as a chimera of the DBD and

a) a σ54 activator domain
b) a library a real or putative σ54 activator domains
c) a library of sequences or domains
d) a genomic library

18) A target ligand identified by method 12) and 13)

19) A DNA binding protein and/or target site identified by method 1) - 10)

20) A protein and/or its target ligand identified by method 1) - 10)

21) An activator of s54 promotors identified by method 17)

Figure 1

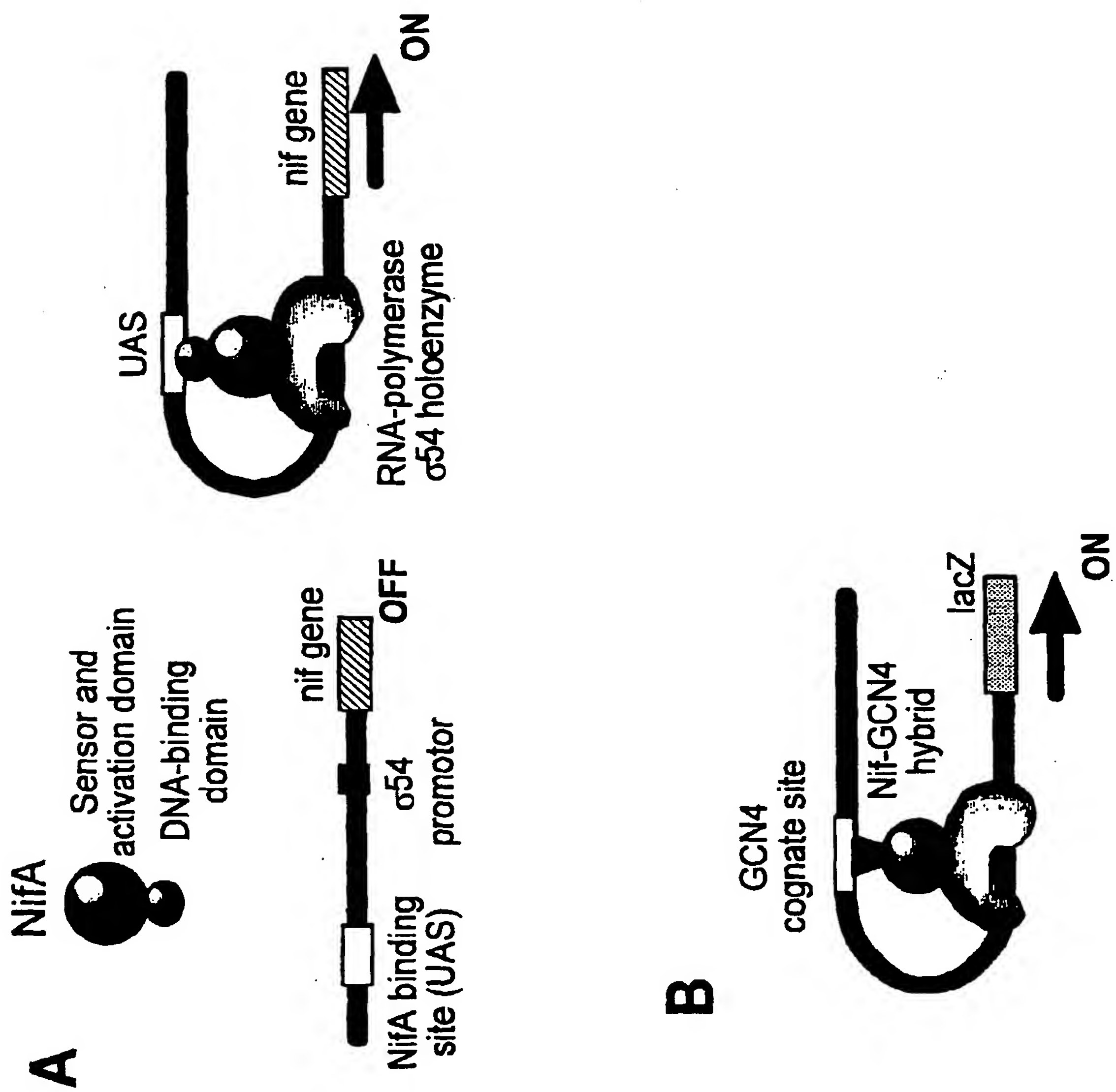
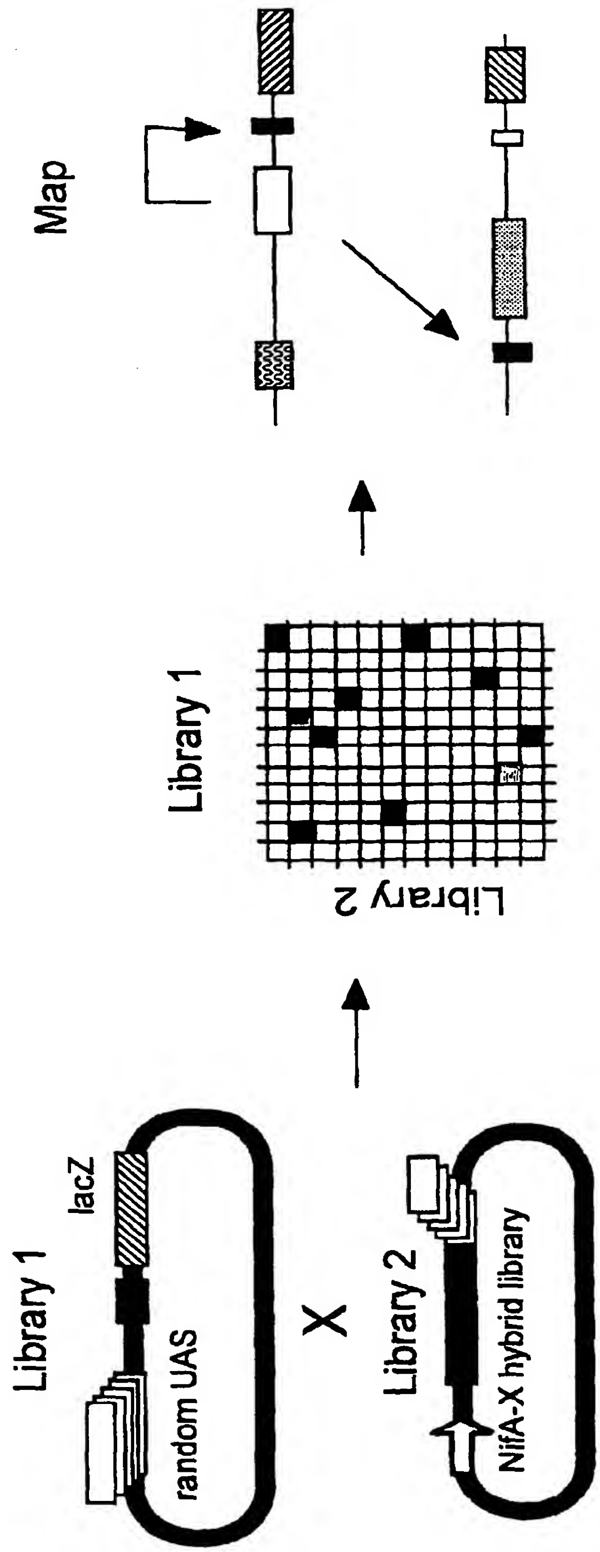

A
NifA
Sensor and activation domain
DNA-binding domain
nif gene
OFF
NifA binding site (UAS)
σ54 promotor
UAS
RNA-polymerase σ54 holoenzyme
nif gene
ON
B
GCN4 cognate site
Nif-GCN4 hybrid
lacZ
ON

Figure 2


Library 1
lacZ
random UAS
X
Library 2
NifA-X hybrid library
Library 1
Library 2
Map

PCT/GB00/03450

D. Young & Co

8/9/00